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New record of *Beauveria pseudobassiana* from Morocco

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Abstract—Two species of *Beauveria* were identified from isolates obtained from endemic *Argania spinosa* forests in Morocco, using both morphological characteristics and molecular data. Although isolates exhibited similar reproductive structures, ITS-rDNA based phylogenetic analysis grouped the Moroccan isolates into two clades with known sequences of either *B. bassiana* or *B. pseudobassiana*. Morphological comparison of the colonies distinguished the two groups of isolates, in full agreement with the ITS phylogenetic analysis. *Beauveria pseudobassiana* is recorded for the first time in Morocco.

Key words—*Ascomycetes,* entomopathogenic fungus, taxonomy

Introduction

Beauveria Vuill. (*Cordycipitaceae*, *Hypocreales*) is a genus of cosmopolitan entomopathogenic fungi found in various types of habitats and ecosystems (Meyling & Eilenberg 2007). Its species are known to infect a wide range of insects from many different orders in natural and agricultural environments (Roy et al. 2010). Preparations from *B. bassiana* have been developed and commercialized as biological control agents (Zimmermann 2007). Other *Beauveria* species hold great potential for the discovery of mycoinsecticidal effect (Thomas & Read 2007) and the production of different bioactive and pathogenetic compounds (Ames & Walsh 2010). Traditionally *Beauveria* species have been identified principally by conidial morphology. However,

the extensive overlap in morphological characters complicates and limits their usefulness for species identification. Furthermore, the discovery of cryptic species using molecular markers (Rehner & Buckley 2005, Ghikas et al. 2010, Rehner et al. 2011, Meyling et al. 2012) makes species identification a challenge within the genus (Fisher et al. 2011). Accordingly, accurate identification, including phylogenetic analyses, has been significantly improved using molecular methods (Rehner et al. 2011).

Through ITS and EF1-alpha sequence analyses, Rehner & Buckley (2005) demonstrated that the morphospecies *B. bassiana* was not monophyletic but comprised two morphologically indistinguishable clades that have been recently recognized as separate species (Rehner et al. 2011, Meyling et al. 2012). Recently, robust molecular phylogenies based on multi-loci sequences have resolved 12 well-supported terminal lineages within *Beauveria* (Rehner et al. 2011). Similarly, recent sequence analyses have resulted in the recent description of four new species (Zhang et al. 2012, Chen et al. 2013, Agrawal et al. 2014, Robène-Soustrade et al. 2015).

In a previous study of entomopathogenic fungi in forests of endemic *Argania spinosa* (L.) Skeels (*Sapotaceae*) in Morocco, *Beauveria* and *Metarhizium* species were found but only a few isolates were identified as *B. bassiana* using molecular analysis of the internal transcribed spacer regions of ribosomal DNA (ITS-rDNA) sequences (Imoulan et al. 2011). The present study aims to adapt the phylogenetic species recognition concept (Rehner et al. 2011) by identifying the strains obtained from the Moroccan forests based on both morphological and molecular analyses.

Materials & methods

Fungal isolates, growth conditions, and morphological observation

Beauveria isolates were recovered from soil samples collected from *Argania spinosa* forests in Morocco using a *Galleria mellonella* baiting method and maintained on Potato Dextrose Agar (PDA) slants at 4°C in complete darkness (Imoulan et al. 2011). The designation of isolates and their geographical origin are given in Table 1. To establish monosporic cultures, conidial suspensions of 1×10^5 conidia ml^{-1} were prepared from fungal cultures grown on PDA for 2 weeks and plated on PDA plates. The single colony propagated from single conidia was transferred into a new PDA dish and incubated at 25°C. Dried agar culture vouchers were deposited in Herbarium Mycologium, Chinese Academy of Sciences, Beijing, China (HMAS).

Microscopic measurements of conidia were taken from slide-cultures produced by inoculating a small amount of mycelium on a block of the appropriate nutrient agar overlaid by a cover slip. Images were acquired using an AxioCam MRc digital camera on a Zeiss AxioScope microscope. Microscopic measurements were performed with

Table 1. *Beauveria* and *Cordyceps* strains and ITS sequences used in phylogenetic analysis. GenBank numbers in bold are newly generated sequences.

Species	Strain	Locality	Host/Substrate	GenBank no.
B. pseudobassiana	2706	Morocco	Soil	**KU364339**
	2629	Morocco	Soil	**KU364340**
	2634	Morocco	Soil	**KU364341**
	2640	Morocco	Soil	**KU364342**
	2649	Morocco	Soil	**KU364343**
	2650	Morocco	Soil	**KU364344**
	2653	Morocco	Soil	**KU364345**
	2659	Morocco	Soil	**KU364346**
	2660	Morocco	Soil	**KU364347**
	2661	Morocco	Soil	**KU364348**
	2687	Morocco	Soil	**KU364349**
	2689	Morocco	Soil	**KU364350**
	2645	Morocco	Soil	**KU364351**
	ARSEF1855	Canada	*Coleoptera*: *Scolytidae*	HQ880796
	ARSEF3405 (T)	USA	*Lepidoptera*: *Tortricidae*	AY532022
	ARSEF6229	China	*Coleoptera*: *Scolytidae*	HQ880799
B. bassiana	2721	Morocco	Soil	**KU364352**
	2718	Morocco	Soil	**KU364353**
	ARSEF1040	Japan	*Lepidoptera*: *Bombycidae*	AY531972
	ARSEF751	Vietnam	*Coleoptera*: *Chrysomelidae*	AY532045
	ARSEF1564 (T)	Italy	*Lepidoptera*: *Arctiidae*	HQ880761
B. australis	ARSEF4622	Australia	*Orthoptera*: *Acridiidae*	HQ880788
	ARSEF4598 (T)	Australia	Soil	HQ880789
B. brongniartii	ARSEF617 (T)	France	*Coleoptera*: *Scarabaeidae*	HQ880776
	ARSEF7058	USA	*Hymenoptera*: *Formicidae*	HQ880773
	ARSEF7517	Japan	*Coleoptera*: *Scarabaeidae*	HQ880767
B. asiatica	ARSEF4850 (T)	Korea	*Coleoptera*: *Cerambycidae*	AY531936
	ARSEF4384	China	*Coleoptera*: *Scarabaeidae*	AY532026
B. sungii	ARSEF1685 (T)	Korea	*Coleoptera*: *Scarabaeidae*	AY531990
	ARSEF7279	Korea	*Coleoptera*: *Scarabaeidae*	HQ880813
B. malawiensis	ARSEF7760	Malawi	*Coleoptera*: *Cerambycidae*	DQ376247
	BCC17613	Australia	—	HQ880824
B. vermiconia	ARSEF2922 (T)	Chile	Soil	AY532012
B. caledonica	ARSEF2251	Brazil	*Coleoptera*	AY532003
	AESEF2567 (T)	Scotland	Soil	AY532006
B. amorpha	ARSEF2641 (T)	Brazil	*Hymenoptera*: *Formicidae*	AY532008
	ARSEF4149	Australia	*Coleoptera*: *Scarabaeidae*	HQ880804
B. rudraprayagi	MTCC8017 (T)	India	Silkworm	JQ266173
B. varroae	ARSEF8257 (T)	France	*Acari*: *Varroidae*	HQ880800
	ARSEF8259	France	*Acari*: *Varroidae*	HQ880801
B. sinensis	RCEF3903 (T)	China	*Lepidoptera*: *Geometridae*	HQ270152
B. lii	RCEF5500 (T)	China	*Coleoptera*: *Coccinellidae*	JN689372
B. kipukae	ARSEF7032 (T)	USA	*Homoptera*: *Delphacidae*	HQ880803
C. militaris	ARSEF5050	USA	*Lepidoptera*	HQ880829

AxioVision Rel. 4.6 software (Zeiss, Welwyn Garden City, UK). Length : width ratios are given as Q, and mean values are indicated by L^m (length), W^m (width), and Q^m (Q).

Genomic DNA extraction, PCR and sequencing

Isolates recovered from single conidia were grown as mycelia for 2 weeks in 250-ml flasks containing 100ml of potato dextrose broth. Cultures were then shaken at 150 rpm on a rotary shaker at 25°C for 7d in darkness. The mycelial samples were pelleted from liquid cultures by centrifugation for 10 min at 3500 rpm (Eppendorf AG Centrifuge, Hamburg), subsequently washed twice with sterile double distilled water, and then lyophilized and stored at –80°C.

For each isolate, 400–500mg of lyophilized mycelium was ground in a sterile mortar using a pestle. Total genomic DNA was extracted following the modified CTAB method described by Yao et al. (1999). The mixtures were incubated for 2 hours at 65°C, then extracted twice with 24 : 1 chloroform : isoamyl alcohol, and centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was transferred to a clean tube and mixed with 1/10 volume of sodium acetate (3 M, pH 5.2). Total genomic DNA was precipitated by adding 2/3 (v/v) of cold isopropanol and chilled overnight at –20°C. DNA was recovered by centrifugation at 10,000 rpm, 4°C for 10 min and washed twice with 70% alcohol, dried at room temperature and resuspended in TE buffer (10Mm Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0). The extracted DNA was stored at –20°C until use.

Genomic DNA was used as template for PCR amplification of ITS region using universal primers ITS5/ITS4 (White et al. 1990). The PCR reactions were performed in a final volume of 50 µl containing 25µl 2× Taq PCR Master Mix (Tiangen Biotech Co., Ltd, China), 0.5 µl of each primer (10 µM), 1 µl of genomic DNA and 23 µl of RNase-Free water. The PCR reactions were performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems) as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 45 s and final extension step of 72°C for 8 min. PCR products were checked by electrophoresis on an agarose gel (1%) at 100 V in 0.5× TAE buffer (40 mM Tris-Acetic acid, pH 8.0, 1 mM EDTA), and sequenced with the same primer pairs. The sequencing was performed at Beijing Genomics Institute (Beijing, China) by using an Applied Biosystems 3730 Analyzer capillary sequencer (Foster City, CA, USA). All sequences generated in this study (KU364339–KU364353) were submitted to GenBank (Table 1).

Phylogenetic and data analyses

Only two (of 100) *B. bassiana* strains sequenced for ITS from Morocco were used as representatives, while all Moroccan strains identified as *B. pseudobassiana* were included in the analyses. Also included were 29 representative *Beauveria* sequences and one sequence of *Cordyceps militaris* (ARSEF 5050; as outgroup) retrieved from GenBank (Table 1). The sequences were aligned using ClustalW Multiple alignment tool (Thompson et al. 1997) and edited manually to avoid some obvious misalignment using BioEdit ver. 7.2.5 (Hall 1999).

The sequence data set was analyzed for maximum parsimony (MP) and Bayesian inference (BI). MP analyses were conducted with PAUP* 4.0b10 (Swofford 2003) and

executed with 1000 replicates of heuristic search option of random sequence additions with branch swapping algorithm by tree bisection reconnection (TBR). Alignment gaps were treated as missing data, and all characters were unordered and equally weighted. Branch support was estimated by bootstrap analysis with 1,000 replicates (Felsenstein 1985) executing the same search strategy as described above. Bayesian inference (BI) analyses were carried out in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) using a general time-reversible model and gamma-distributed rate variation to account for rates of variation across sites, with a proportion of invariant sites. The remaining parameters were default values. One tree was saved every 1,000 generations from a total of 5,000,000 Markov chain Monte Carlo (MCMC) generations; the first 25% of the trees were discarded as burn-in. Clades with bootstrap values for MP ≥50% and Bayesian posterior probability ≥70% are labeled above the nodes in the phylogenetic tree.

Results

Morphological observation

Microscopically *Beauveria* isolates from Morocco appeared typical to those described elsewhere (e.g., Humber 1997, Rehner et al. 2005). The colony characteristics were: white or pale-yellow mycelium closely (or not) appressed to agar surface, becoming farinaceous during sporulation and the reverse side uncoloured or yellowish white (Figs 1, 2). Conidiophores appeared like dense spherical clusters of subglobose to flask-shaped conidiogenous cells extending upward with an elongating sympodial denticulate rachis (zigzag appearance) giving rise to sessile, hyaline, holoblastic smooth conidia (Figs 3, 4). Two isolate groups were morphologically distinguished based on the colony colour as shown by aerial hyphae during conidiation: off-white and loosely floccose vs. yellowish-brown and densely farinaceous (Figs 1, 2).

Phylogenetic analyses

The amplification of ITS-rDNA region produced a uniform fragment size of 560bp. Using Blast search, the sequences exhibited high sequence homology to those named *B. bassiana* or *B. pseudobassiana* in GenBank.

The ITS dataset comprised 571 characters after the exclusion of ambiguous positions from both ends. There were 488 constant, 37 parsimony-uninformative, and 46 parsimony-informative characters. The heuristic search yielded six most-parsimonious trees with tree length (L) = 139, consistency index (CI) = 0.719, homoplasy index (HI) = 0.281, retention index (RI) = 0.807. MP and BI phylogenetic analyses produced trees with similar topologies that resolved most *Beauveria* lineages in separate terminal branches (Fig. 9). Independently of the algorithm used for phylogenetic analyses, *B. brongniartii* and *B. australis* were not well distinguished as unique clusters based on ITS-rDNA sequences.

Moroccan strains of *Beauveria* were grouped into two individual terminal clades corresponding to *B. bassiana* and *B. pseudobassiana* with MP bootstrap support ≥76% and BI posterior probabilities ≥99% (Fig. 9). These two groups corresponded well to their colony colours, off-white in the *B. bassiana* clade and yellowish-brown in the *B. pseudobassiana* clade.

Taxonomy

Imoulan et al. (2011) previously reported *Beauveria bassiana* from Morocco. Here we describe *B. pseudobassiana* as a new record for Morocco.

Beauveria pseudobassiana S.A. Rehner & Humber, Mycologia 103: 1068 (2011) Figs 2–8

Colonies growing fairly well on PDA at 25°C in the dark, reaching a diameter of 28–37 mm after 7 days incubation, and attaining a diameter of 36–42mm in 10 days; velutinous to cottony with an overgrowth of aerial hyphae up to the agar surface, white first and changing to yellowish-brown or pale yellow; becoming less powdery in older cultures and rarely with a dense farinaceous surface layer. Reverse side yellow with white margin. Vegetative hyphae septate, branched, hyaline, smooth-walled, 1–3 µm wide. Conidiogenous cells solitary but usually consisting of dense lateral clusters, base subspherical to flask-shaped (sometimes ampulliform), 2.0–9.0 × 1.5–3 µm, apex with an indeterminate denticulate rachis, produced laterally on aerial hyphae or from subtending cells. Conidia globose to subglobose, 2–4 × 1–3.5 µm, Q = 1–2.5 (L^m = 2.6 µm, W^m = 2.1 µm, Q^m = 1.4), rarely ellipsoid; hyaline, walls thin and smooth; produced from conidiogenous cells and occasionally directly from hyphal tips or laterally from hyphae.

Material examined: **MOROCCO**, **Oujda**; from soil under *Argania spinosa* tree, 23 March 2009, coll. A. Imoulan, strain numbers 2629, 2634, 2640, 2645, 2649, 2650, 2653, 2659, 2660, 2661 (HMAS 254118–254127, dried agar cultures); Tamanar, 28 March 2009, coll. A. Imoulan, strain numbers 2687, 2689 (HMAS 254128–254129, dried agar cultures); Amskroud, 29 March 2009, coll. A. Imoulan, strain number 2706 (HMAS 254131, dried agar culture).

Remarks: *Beauveria pseudobassiana* from Morocco differed from *B. bassiana* by its colony surface, which is yellowish-brown to pale yellow (Fig. 2) becoming less powdery when old, and by its occasional production of ellipsoid conidia (Fig. 7). The colony surface of *B. bassiana* is usually white to off-white

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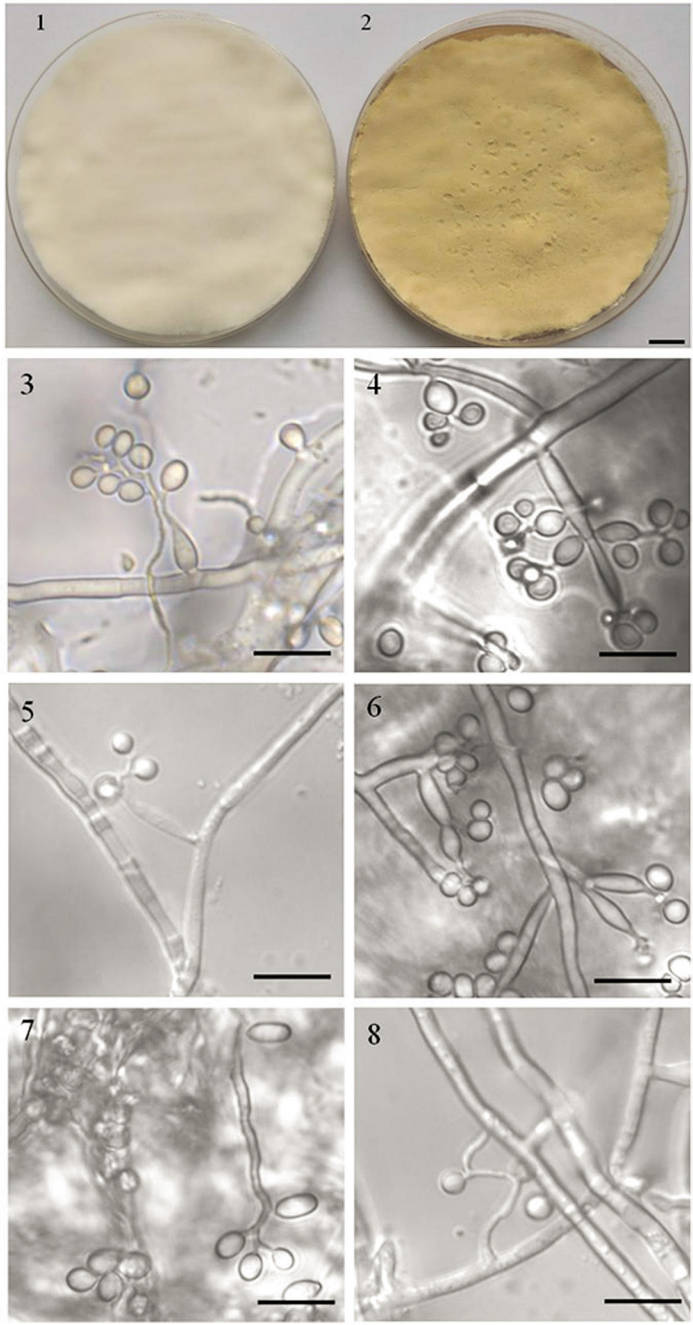
Figs 1–8. ***Beauveria bassiana*** (strain 2718): 1. colony and aerial hyphae. ***Beauveria pseudobassiana*** (strain 2629): 2. colony and aerial hyphae; 3–8. conidiophores, conidiogenous cells, and conidia — note conidiogenous cell produced from subtending cells (6), conidia forming from a hyphal tip (7), and conidia produced directly from mycelium (8). Scale bars: 1, 2 = 10 mm; 3–8 = 5 µm.


1
2
3
4
5
6
7
8

(Fig. 1) becoming powdery when old, owing to the production of a large number of globose to subglobose conidia.

Discussion

The study of mycology in Morocco is less developed with few works being published on Moroccan fungi, particularly on entomopathogenic fungi (Imoulan et al. 2011, Imoulan & El Meziane 2013). The new species record for Morocco reported here demonstrates that *B. pseudobassiana* occurs in *A. spinosa* forests along with other entomopathogenic fungi such as *B. bassiana*, *Metarhizium anisopliae,* and *Paecilomyces lilacinus* identified previously by Imoulan et al. (2011).

As shown in the most recent taxonomic revision based on the phenotypic criteria combined with molecular evidence (Rehner & Buckley 2005, Rehner et al. 2011), *B. pseudobassiana* and *B. bassiana* comprise a morphospecies group, morphologically indistinguishable yet phylogenetically only distantly related. Interestingly, our morphological observation nonetheless revealed that characters of both colony and conidial spores were sufficient to discriminate the two species. As these morphological distinctions have not been mentioned elsewhere, it is unclear whether our experience represents an unusual case or if it is possible to find additional characters to distinguish *Beauveria* species morphologically. It would be very useful if such characters could support DNA-based phylogenies in *Beauveria.*

In the current study, ITS-rDNA-based phylogenetic analyses succeed in distinguishing *B. bassiana* and *B. pseudobassiana* among the species analyzed. Because *Beauveria* includes cryptic species that cannot be resolved solely by ITS analyses, multilocus-based phylogenies are becoming increasingly important for accurate assignment of strains to specific clades. Molecular phylogenies involving multiple genes of translation elongation factor-1α (TEF), RNA polymerase II largest subunit (RPB1), RNA polymerase II second largest subunit (RPB2), and the Bloc nuclear intergenic have become well-established for determining unique terminal lineages for all *Beauveria* species (Rehner et al. 2011, Zhang et al. 2012, Chen et al. 2013, Agrawal et al. 2014, Robène-Soustrade et al. 2015). However, our ITS sequence analyses performed well in distinguishing 12 of the 15 species included, with *B. australis* and *B. brongniartii* grouping in a single unresolved clade, and *B. rudraprayagi* (MTCC8017) grouping with *B. pseudobassiana* (Fig. 9). Although the additional genes are required in resolving closed related species, ITS sequence is still powerful in identifying most of the known species of *Beauveria* as shown in Fig. 9.

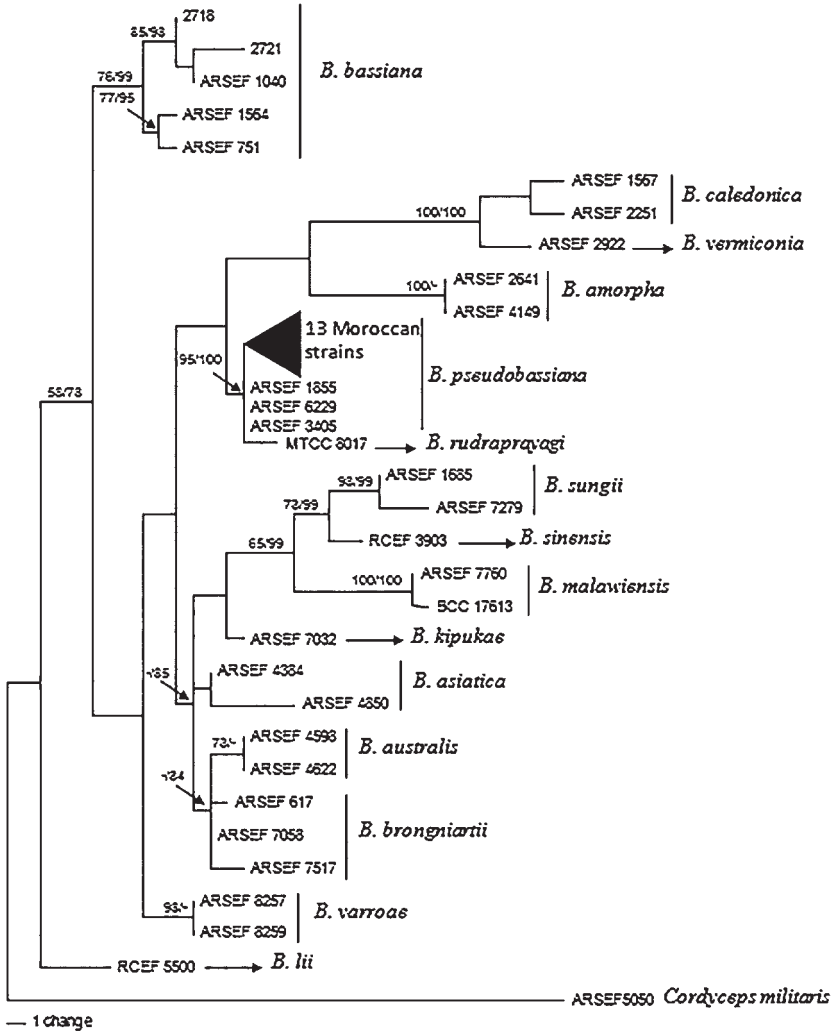


Fig. 9. Phylogenetic tree of *Beauveria* based on Maximum parsimony and Bayesian inference of the ITS-rDNA sequences. Bootstrap values ≥50% and posterior probabilities ≥70% are labeled above branches and separated by /. Terminal clades are labeled according to ARSEF accession numbers of individual isolates reported in Rehner et al. (2011) except RCEF5500 and RCEF3903 (Zhang et al. 2012, Chen et al. 2013).

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